Inhibitory activity of tannin-containing crude drugs on recombinant hepatits C virus protease.

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Abstract

Aiming at finding novel hepatits C virus (HCV) drugs having easier access and requiring less economical burden, we studied on crude drugs for anti HCV protease activity. Thirteen crude drugs which are known rich in tannins were selected, since we had found peel and seeds of a tropical fruit in Peru, Camu camu, and their tannic ingredients were effectively inhibited the recombinant HCV protease. Methanolic extracts of the crude drugs were tested for inhibitory activity on the enzyme reaction, and 6 of them at a concentration of 100 μg/ml inhibited the reaction more than 90 %. Five of these effective extracts contained as much as 800-1500 μgGAE/ml phenolics, but the extract of kaki calyx (KC) contained less than 400 μgGAE/ml. On the other hand, that of gambir (Ga), which contained more than 1300 μgGAE/ml phenolics, did not effectively inhibit the reaction. We now focus on the effective ingredients contained in the extract of KC.

Key words : HCV, protease, inhibitor, crude drug, phenolics

Introduction

Hepatitis C virus (HCV) is a major cause of acute and chronic human hepatitis. Approximately 3 % of the world population (180 million people worldwide) is infected with HCV [1]. Cure rate has risen to 45-75 % by the current standard treatment with combination of pegylated interferon (Peg-INF) with ribavirin (RBV). Moreover, by the addition of anti-protease inhibitors to the standard treatments, 70-95 % of complete response is expected[2-5]. In spite of development of such successful anti-HCV therapies, many patients with HCV in the world cannot reach these advanced medical treatments, and are anticipated alternative treatments having easier access and requiring less economical burden. Folk medicines of various areas in the world are hopeful candidate for developing novel HCV drugs to meet such demands [6]. We reported that anti-HCV protease activity of an extract of a tropical fruit in Peru peel and seeds. Furthermore, its tannic ingredients supposed to be the active principles [7]. We, therefore, focus on crude drugs which are known rich in tannin for their HCV protease activity using the recombinant enzyme.

Materials and methods

Extract preparation from crude drug material.

Powdered crude drugs (Table 1) were obtained from Mikuni Co Ltd. The crude drug powders were kept in a desiccator for 48 hours for drying. Ten or 5 g of each crude drug powder was suspended in a 100 ml methanol and subjected to ultrasonic wave irradiation for 30 min. The methanol suspension was kept still, and the resulted clear methanol phase was obtained. The suspension in fresh methanol / ultrasonic irradiation was repeated two more times. All the methanol phases were combined and filtered, and the methanol was removed by evaporation. The remaining residue was

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Table 1 Crude drugs used and yield of extraction

Abbreviation	Crude drug	生薬名	Original plant source	Ccude drug (g)	Extract (mg)
AS	Arecae Semen	ビンロウジ	Areca catechu L	10.3	80.9
CF	Chebulae Fructus	訶子	Terminalia chebula Reitus	10.1	18.8
CR	Clematidis Radix	威霊仙	Clematis chinensis Osbeck	5.1	31.1
Ga	Gambir	アセンヤク	Uncaria gambit Roxburgh	5.0	23.2
GH	Galla Halepensis	没食子	Quercus infectoria Olivier + Cynips tinctoria Hartig	10.0	69.1
Gu	Guava	蕃石榴	Psidium guajava L.	10.2	73.1
KC	Kaki Calyx	柿蒂	Diosphyros kaki Thunb.	10.1	37.3
MC	Myricae Cortex	揚梅皮	Myrica rubra Sieb. et Zucc	10.2	59.3
QF	Quisqualis Fructus	使君子	Quisqualis indica L	10.0	42.5
SM	Salviae Miltiorrhizae Radix	丹参	Salvia miltiorrhiza Bunge	10.1	12.0
TP	Trapae Pericarpium	菱果皮	Trapa japonica Flerov	5.0	25.9
UU	Uvae Urusi Folium	ウワウルシ	Arctostaphylos uvae-ursi Sprengel	5.1	70.4
VH	Visci Herba	桑寄生	Viscum album L var coloratum (Komar) Ohwi	10.1	25.6

weighed, and dissolved in DMSO to a concentration of 100 mg/ml. The DMSO solution was diluted to 2.0 mg/ml and 0.2 mg/ml.

Recombinant HCV protease (ΔNS3) preparation.

An expression plasmid containing the amino acid sequence of HCV polypeptide from 1027 to 1218 and a six histidine residues in series at N-terminal adjoining region to the HCV peptide in Escherichia coli JM109 was expressed according to the method reported previously [8]. The bacterial pellet was yielded by centrifugation and stored at -30 ℃. The expressed recombinant peptide with His-tag was purified using Ni-NTA Quick Start Kit (QIAGEN), and the purity of the peptide was estimated more than 80 %.

Enzyme reaction

The recombinant enzyme together with the HCV NS4 peptide P41 hovering amino acid 1673-1692, which was found cooperative for the proteolytic reaction, were reacted with a synthetic peptide S-1 (DDIVPC / SMSYTWT) corresponding the cleavage site of HCV NS5A/5B [9]. The standard enzyme reaction of ΔNS3 was performed as followed: ΔNS3 (0.74 μM) and P41 (0.43 mM) were incubated in a buffer (Tris-HCl (pH 7.8), 30 mM NaCl, 5 mM CaCl2, 10 mM DTT) at 37 ℃, and the reaction was started by addition of S-1 (85 μM). The reaction was prolonged for 15 min, and stopped by heating at 95 ℃ for 1 min. The assays were carried out in triplicate.

HPLC analysis

The HPLC analysis was made on a Shimadzu liquid chromatography system (LC-10ADvp Pumping Unit, DGU-14AM degasser, SPD-10A UV–VIS ultraviolet detector, CTO-10ACcolumn oven, Chromatopac C-R8A data processor, and SLC-10A System Controller) equipped with a Mightysil RP-18 Aqua 250-4.6 (5 μm) (Kanto Kagaku). The column was eluted with 23 % acetonitrile in 0.05 % trifluoroacetic acid at 40 ℃. The UV signal at 210 nm of the cleaved product and the substrate was detected. Inhibition (%) was calculated based on the HPLC area of the remaining substrate of a reaction (Rn), that of positive control (PC), the reaction carried out without extract, and the negative control (NC), the reaction without enzyme and extract, as the following:

Inhibition (%) = 100 x (Rn-PC)/(NC-PC)

Total phenolics determination

The total phenolics was determined colorimetrically by the modified method of Folin-Ciocalteau [10]. Samples (40 μl) were mixed with 5-fold diluted Folin-Ciocalteau reagent (0.5 ml), 10 % sodium carbonate (0.5 ml) and water (1 ml), and were incubated for 2 hr at room temperature. Absorbance at 760 nm was measured. The total phenolic content of the samples was expressed as μg gallic acid equivalent (GAE). The assays were carried out in triplicate.

Result and discussion

The term of “tannin” is based on historical use of polyphenolics from plants that has been used for

tanning of animal hides into leather. They have bitter and astringent taste, and show tendency of interaction with proteins and metals.

Thirteen crude drugs, which are known rich in tannin, were extracted with methanol. The abbreviations, original plants, and yields of the extract were summarized in Table 1. Their inhibitory activity on the recombinant HCV protease was examined as shows in Fig 1. Six of these extracts, AS, CF, GH, KC, MC, and TP, at a concentration of 100 μg/ml inhibited the enzyme reaction more than 90 %. In addition, the extracts of AS and GH at a concentration of 10 μg/ml inhibited the reaction approximately 50 %. Total phenolics content was shown in Fig 2.

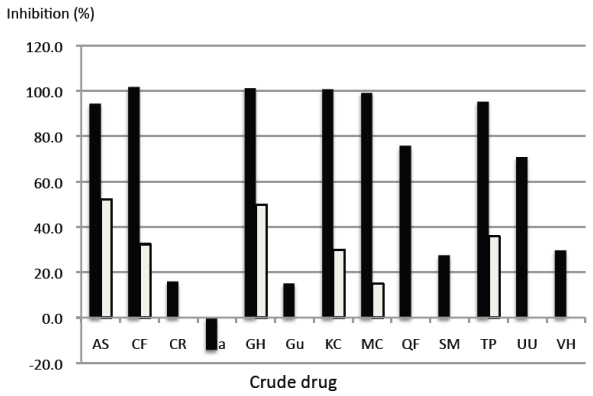

Fig 1 Inhibition of enzyme reaction.

Closed and open bars indicate inhibition at the concentration of 100 μg/ml and 10 μg/ml, respectively. Values are means of triplicate assays.

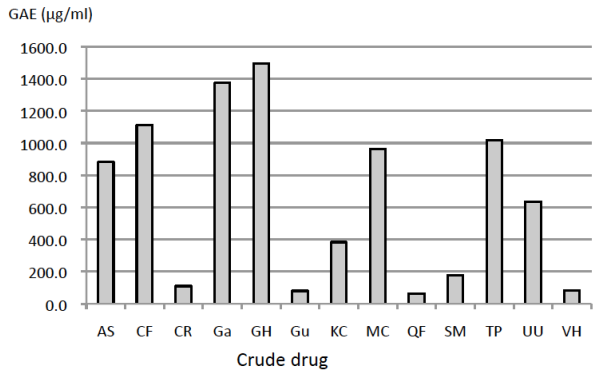

Fig 2 Phenolic content of crude drug extracts.
Values are means of triplicate assays.

The extracts with higher inhibitory activity AS, CF, GH, MC, and TF contained relatively higher phenolics content of 800 – 1500 μgGAE/ml. However, Ga, which contained as much as 1300 μgGAE/ml, did not show inhibitory activity at the concentration of 100 μg/ml. On the contrary, KC, which contained phenolics less than 400 μgGAE/ml, showed relatively high inhibitory activity of approximately 100 % and 30 % at the concentrations of 100 μg/ml and 10 μg/ml, respectively. The result suggests that some phenolics are contributed to inhibition on enzyme, but all of phenolics are not necessarily inhibitory. AS contains approximately 15 % of arecatannins, which is a class of condensed tannins containing epicatechins[11]. CF is rich in hydrolysable tannins, such as chebulagic acid, chebulinic acid, punicalagin[12]. On the other hand, Ga contains mainly smaller phenolics such as catechins and epicatechins, which were not very effective inhibitors for the enzyme reaction[13]. GH contains pentagalloyl glucose (PGG) 50 – 70 % [14], and PGG has IC50 8.5 μM for the HCV protease inhibition [13]. KC is reported to contain fatty acids, triterpenoids and flavonols[15]. The fruits and leaves of persimmon, on the other hands, are reported to have polyphenolics[16]. Ingredients of TP are not well known. Therefore, we are now concentrating on the KC, and their active ingredients for the HCV protease inhibition.

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タンニン生薬の組み替えC型肝炎タンパク分解酵素阻害活性

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要　旨

C型肝炎ウイルス（HCV）感染者は、世界に１億人以上いるとされている。先進国では先端的な医薬品の開発で、HCVを病原とする慢性及び急性C型肝炎の治癒率は飛躍的に高まった。一方、世界には経済的に、また医療体制の問題から、それら先端医薬品を手に入れ難い地域も多い。我々は安価で、また手に入れやすいC型肝炎治療薬を開発するために、各地の伝統薬物（生薬）に着目した。特にタンニンを多く含んだ13生薬を選び、メタノールエキスを作成した。HCVの感染に必須であるHCVタンパク質分解酵素を、大腸菌で生成し、この組み換え酵素に対する阻害効果をスクリーニングした。13のエキスのうち６のエキスの100 μg/mlに、90％以上のHCVタンパク質分解酵素阻害効果を認めた。ポリフェノール含量の高いエキス（800-1500 μgGAE/ml）が阻害効果も高い傾向であったが、阿仙薬エキスはポリフェノール含量が高い（1300 μgGAE/ml以上）にも係らず、ほとんど阻害効果がなかった。一方柿蒂エキスは、ポリフェノール含量は比較的低かったが（400 μgGAE/ml以下）、高い阻害活性が認められ、ポリフェノールの種類によっても、阻害活性が異なることが示唆された。今後柿蒂の活性成分について、さらに詳しく研究する。

キーワード：HCVタンパク分解酵素，阻害剤，生薬，フェノール類